NEWBORN SCREENING STRATEGIES FOR DISORDERS OF CREATINE METABOLISM

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Creatine is necessary to transfer energy between cellular compartments. Creatine is converted to phosphocreatine by the creatine kinase reaction within mitochondria and phosphocreatine generates adenosine triphosphate (ATP) in the cytoplasm. ATP powers most of the energy consuming reactions in cells. Defects in creatine synthesis or transport disrupt this process and result in brain creatine deficiency syndromes. Affected patients have developmental delay, hypotonia, autism, seizures, and impaired motor skill development. Defects of creatine synthesis are caused by impaired activity of the enzymes guanidinoacetate methyltransferase (GAMT) and arginine:glycine amidinotransferase (AGAT), both transmitted as autosomal recessive traits, whereas defect in creatine transport (*SLC6A8* Gene) are transmitted in an X-linked recessive manner. Patients with defects in creatine synthesis respond to creatine supplementation and dietary manipulations. This therapy is more effective if initiated before mental retardation is evident. For this reason, diagnosis should be accomplished as soon as possible with newborn screening. Here we report a reliable three-tier testing method for screening for GAMT and AGAT deficiency in newborns'

blood spots. Creatine and guanidinoacetate are detected in newborn screening blood spots by tandem mass spectrometry (MS/MS). Second-tier testing using LC-MS/MS confirms more quantitatively low creatine and increased or decreased guanidinoacetate levels, while third-tier testing consists of DNA sequencing to identify mutations in the *GAMT* and *AGAT* Genes. This test can potentially identify newborns with GAMT and AGAT deficiencies with low false positive rate and could be applied to newborn screening nationwide.

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CHAPTER I

INTRODUCTION

Newborn screening began in the 1960s when a test for a metabolic disorder, phenylketonuria (PKU), was developed by Robert Guthrie (Newborn Screening 2011). Guthrie knew that treatment for PKU is more effective if initiated at the earliest date possible and could prevent mental retardation. This general concept applies to many other disorders, including the defects of creatine synthesis GAMT and AGAT deficiencies (O'Rourke, et al., 2008, Battini, et al., 2006). Newborn screening could potentially detect these conditions and prevent irreversible damages that occur without treatment.

Creatine, or α-N-methylguanidino acetic acid, is an amino acid necessary for energy metabolism. Creatine was initially recognized as deriving from meat (*kreas* in Greek meaning flesh) in 1832 (Longo et al., 2011). Creatine is phosphorylated to phosphocreatine that can release phosphate to yield energy and convert ADP to ATP (Verhoeven et al., 2005). In tissues requiring high levels of energy, such as muscle, brain, and heart, creatine is especially important for normal functioning. Creatine is synthesized by the body in the liver, pancreas, and kidneys (Battini, et al., 2006) and is then distributed to all tissues in the body by the action of specific creatine transporters. Creatine and its

phosphorylated form, phosphocreatine, spontaneously break down to creatinine that is excreted in the urine (Skyut-Cegielska et al., 2004). The creatinine lost in urine excretion is directly proportional to creatine intake (Battini, et al., 2006). Creatinine loss must be restored by new synthesis and dietary intake. In humans, approximately half of the daily creatine required is taken in through diet from meat, fish and dairy products. The remaining creatine needed must be synthesized by the AGAT/GAMT pathway in the body (Braissant, et al., 2010).

GAMT and AGAT Deficiency

Two enzymes are necessary for the synthesis of creatine: guanidinoacetate methyltransferase (GAMT, OMIM 601240) and arginine: glycine amidinotransferase (AGAT or GATM, OMIM 602360) (Almedia, et al. 2004; Longo et al., 2011). Creatine is metabolized in a two-step process (Fig. 1). The first step is the transfer of an amido group from arginine to glycine. This step produces guanidinoacetic acid and ornithine (Skyut-Cegieslska, et al., 2004). The second step in creatine synthesis involves the transfer of a methyl group from S-adenosylmethionine to guanidinoacetate (GAA) to produce creatine and S-adenoslyhomocysteine (Dhar et al., 2008). Once creatine is synthesized, it is transported to the brain and muscle via the blood circulation. Creatine can enter cells and tissues through specific membrane transporters, the most important of which is the sodium and chloride dependent creatine transporter 1 (CT1, CRTR, CRT, OMIM 300036) encoded by the *SLC6A8* Gene (Ardon, et al., 2010).

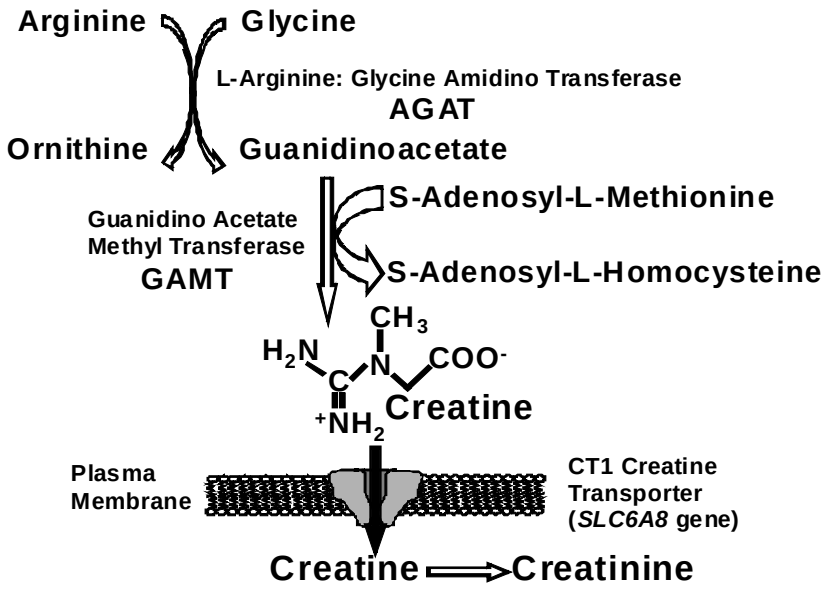


Figure 1. Creatine synthesis and transport. Creatine is synthesized from the amino acids arginine and glycine through the action of the enzymes AGAT and GAMT. AGAT synthesizes guanidinoacetate to which a methyl group is added from S-adenosylmethionine by GAMT to generate creatine. Creatine enters cells and the brain through the CT1 creatine transporter encoded by the *SLC6A8* Gene. Metabolism of creatine leads to formation of creatinine that is excreted in urine. (Longo et al., 2011)

Brain creatine deficiency syndromes are a group of rare disorders that include two recessive conditions that impair the synthesis of creatine (GAMT deficiency, OMIM 612736; and AGAT deficiency, OMIM 612718) or its transfer to the brain (X-linked recessive SLC6A8 creatine transporter deficiency, OMIM 300036)) (Longo et al., 2011) (Fig. 1). These disorders are characterized by brain creatine deficiency, detectable by magnetic resonance spectroscopy (MRS) (Longo et al., 2011; Skyut-Cegieslska, et al., 2004). Affected patients have mental retardation, hypotonia, autism, behavioral problems and seizures (Dhar et al., 2009; Edvardson, et al., 2010; Schulze, et al., 2001).

Their real incidence is unknown, but some studies indicate that up to 2.7% of X-linked mental retardation cases could be due to creatine deficiency syndromes. These conditions were only recently discovered: GAMT deficiency was initially reported in 1994 (Nasrallah, et al., 2010) and AGAT deficiency in 2001 (Verhoeven et al., 2005). Many physicians are unfamiliar with these disorders and confuse their symptoms with other more common conditions. It is also difficult to obtain testing for these disorders because few laboratories offer diagnostic testing (Nasrallah, et al., 2010).

Therapy for the two defects in the biosynthesis of creatine, GAMT and AGAT deficiencies, consists in the administration of creatine supplements. In GAMT deficiency, the synthesis of guanidinoacetate is also prevented by administration of ornithine (the product of the reaction), restriction of arginine (one substrate of the reaction), and administration of benzoate that binds to glycine reducing its levels (glycine is the other substrate of the reaction). With treatment, seizures improve and development progresses. Treatment before symptoms appear has been shown to prevent mental retardation. If an accurate and reliable screening test for these conditions was available, the criteria for inclusion in newborn screening programs would be met. The purpose of the study was to evaluate the feasibility of including additional markers of creatine deficiency syndromes, specifically creatine and guanidinoacetate in the newborn screening test by MS/MS

CHAPTER II

NEWBORN SCREENING AND TANDEM MASS SPECTROMETRY

Newborn screening is a public health activity that started in the early 1960s thanks to Dr. Robert Guthrie, who developed a screening assay for phenylketonuria (PKU) from newborns' blood spotted and dried on filter paper (Newborn Screening 2011). Since then millions of infants in the United States and in the world have been screened for a variety of genetic disorders. In the last ten years tandem mass spectrometry (MS/MS) has been introduced in newborn screening laboratories, allowing multiplex analysis of several analytes from only one sample. The use of MS/MS has allowed the expansion of newborn screening, which now includes 30+ disorders.

The aim of newborn screening is the early identification and treatment of conditions that would not be detected before severe complications, such as irreversible organ damage or death, occur. New conditions are included in a newborn screening program only if certain criteria are met. These criteria evaluate the characteristics of the disease, the test used to screen for it, and the newborn screening program. The disease to be screened must be serious and fairly common. The natural history of the disease must be understood and

treatment must be available. The screening test must be reliable, valid, and affordable.

Most metabolic disorders fit all of these criteria and can be detected in the newborn period by tandem mass spectrometry. Two main classes of metabolites are detected by this technique: amino acids and acylcarnitines. Amino acids become elevated in certain aminoacidopathies (e.g., PKU, tyrosinemia, and maple syrup urine disease), while the study of the acylcarnitine profile can identify defects of fatty acid oxidation (e.g., medium-chain acyl-CoA dehydrogenase deficiency **(**MCAD) and very long-chain acyl-CoA dehydrogenase deficiency(VLCAD)) and organic acidemias (e.g., propionic acidemia, methylmalonic acidemia, and glutaric acidemia type 1).

Tandem Mass Spectrometry Methodology

Tandem mass spectrometry measures the ratio of the mass (m) of a chemical to its charge (z). A small punch (4.7 mm diameter) of whole blood collected on filter paper provides the sample needed for MS/MS analysis. The sample is extracted with methanol containing deuterated internal standards. After drying the extract, amino acids and acylcarnitines are derivatized to butylesters. The derivatized mixture is dried, reconstituted with a solvent that is compatible with the mobile phase, then injected in the mass spectrometer.

All molecules are first ionized, typically by electrospray. The ions formed are then separated according to their mass to charge (m/z) ratios. Since most of the ions have a single positive charge, their mass to charge ratios corresponds to

the masses of the molecules ionized in this process. Two mass spectrometers are used in tandem to separate and analyze mixtures of compounds, such as amino acids and acylcarnitines. After the ions are separated by the first mass spectrometer, they enter the “collision cell” where they are broken down into fragments by collision with a neutral gas. The fragments pass through a second mass spectrometer that separates them according to their mass to charge (m/z) ratio (Kushnir 2010).

The acquisition of data by MS/MS can be accomplished in two different ways. First by class specific analysis where each molecule has a characteristic fragmentation pattern and classes of compounds will fragment in a similar way. For example, all acylcarnitines will produce a similar fragmentation pattern. With the second type of acquisition, target compound analysis, one can derive information about a specific class of compounds. The focus is on one component of the sample. Labeled internal standards (amino acids and acylcarnitines with the same chemical and physical properties of the natural analogues but with higher mass/charge ratio due to the presence of stable isotopes such as deuterium or carbon-13) are added to the extraction mixtures to quantify the different species. The analysis is very fast (<2 minutes) and suitable for high throughput application. With the MS/MS platform, it is also easy to increase the number of analytes detected with minimal additional cost and without requiring additional sample.

Materials

De-identified blood spots, remaining from newborn screening, were used in this study. This study was approved by the IRB of the University of Utah and of the Utah Department of Health. After extraction and derivatization, the samples were injected onto a Waters BEH C18 1.7µm 2.1x100 mm and analyzed in a Waters Xevo TQ MS. The deuterated internal standard d-2-guanidinoacetate was purchased from DCN Isotopes. Hydrochloric acid (3N) in butanol, used for the derivatization step, was purchased from Regis Technologies. Methanol (MeOH) and acetonitrile (ACN) were HPLC grade and purchased from Burdick and Jackson.

Dried Blood Spot Preparation

Packed red blood cells were obtained from Blood Services at ARUP. The blood cells were washed 3 times with a 0.9% saline solution. After the final washing, the hematocrit was measured and it was adjusted to a final value of 55% by diluting the blood cells with serum. This was used to prepare standards and controls. The hematocrit was chosen to mimic the hematocrit observed in newborns.

Standards

Diluted packed red blood cells were used to prepare standards with the same matrix as the samples. For this study we focused on the identification of guanidinoacetate. We prepared guanidinoacetate standards in blood at the

following concentrations: 0, 1µM, 4µM, 20µM, and 40µM. Once the calibrators in blood were prepared, they were spotted on filter paper and allowed to dry. Following the extraction protocol described below, a time course was performed to evaluate the best extraction time for guanidinoacetate.

Extraction and Analysis

The extraction procedure for the first tier test is the same procedure used (at ARUP) for newborn screening to detect amino acids and acylcarnitines (routine screening), with the addition of internal standards for guanidinoacetate and creatine. With the second tier test, samples determined to have an elevated guanidinoacetate by first tier testing, are analyzed using LC-MS/MS. With this system, the chromatographic separation allows detection of possible isobaric interferences (fragments that have the same mass to charge ratio of guanidinoacetate and would not be resolved by MS/MS alone.

CHAPTER III

PROCEDURE

First Tier

This procedure follows the main extraction protocol used for the newborns' spots. Punches (4.7 mm) of dried blood spots (standards, controls, and "normal" newborns' blood spots) were placed in 96-well flat bottom plates; 200 µL of methanol containing internal standards (amino acids, acylcarnitines, succinylacetone, and guanidinoacetate) were added to each well. After a 5 minute incubation at ambient temperature, 100 µL of 3mM hydrazine hydrate in water were added to each well. The plate was placed in an incubator at 37℃ and rotated at 110 revolutions per minute (rpm) for 25 minutes (after evaluating time course results). The supernatant was transferred to 96-well round bottom plates and dried under nitrogen. Butanolic hydrochloric acid (50 µL) was added to the wells to convert analytes into butyl-derivatives. After 15 minutes incubation at 65ºC, the samples were dried under nitrogen, reconstituted with 200 µL of a 50/50 mixture of acetonitrile and water containing 0.02% formic acid, and injected (5µL) in the MS/MS (flow injection).

Second Tier

Punches (4.7 mm) of dried blood spots (standards, controls, and newborns' blood spots) were placed in 96-well flat bottom plates; 200 µL of methanol containing internal standards were added to each well. Then the 96 well plates were covered and put on a shaker, at a medium-high setting, for 15 minutes. The solvent was then transferred from all 96 wells into another 1mL 96 well plate. The plate was dried for 10 minutes under a SPE-Dry 96 Nitrogen evaporator and then allowed to cool for 2 minutes. The samples were derivatized with 100µL of 3N hydrochloric acid in butanol. The plate was shaken at a high setting for 2 minutes. Then it was put into an incubator at 65ºC with a heated metal block on top for 20 minutes. The plate was dried under nitrogen again using the previous conditions. Then the samples were reconstituted with 50µL of 70:30 water/acetonitrile and shaken. Aliquots of the samples were injected (5µL) into the LC-MS/MS.

CHAPTER IV

RESULTS

Time Course

Guanidinoacetate standards prepared in methanol, water, and in blood at several concentrations were spotted on filter paper, dried, and extracted according to the above protocol, using three different extraction times: 15 minutes, 30 minutes, and 45 minutes. The results were compared (Figures 2 and 3). The recovery of guanidinoacetate was independent of the extraction time. The extraction time for our subsequent experiments was set at 25 minutes.

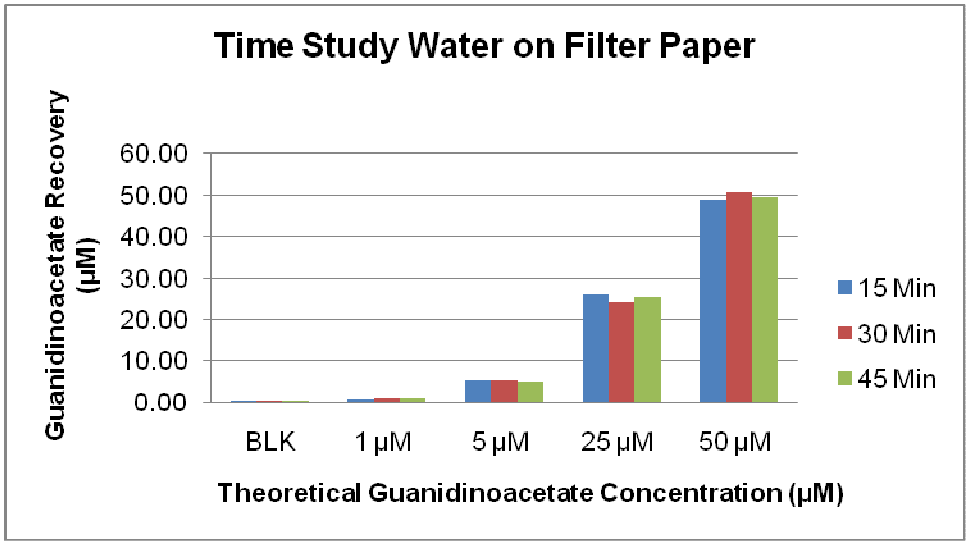


Figure 2. Guanidinoacetate standards prepared in water and spotted on filter paper.

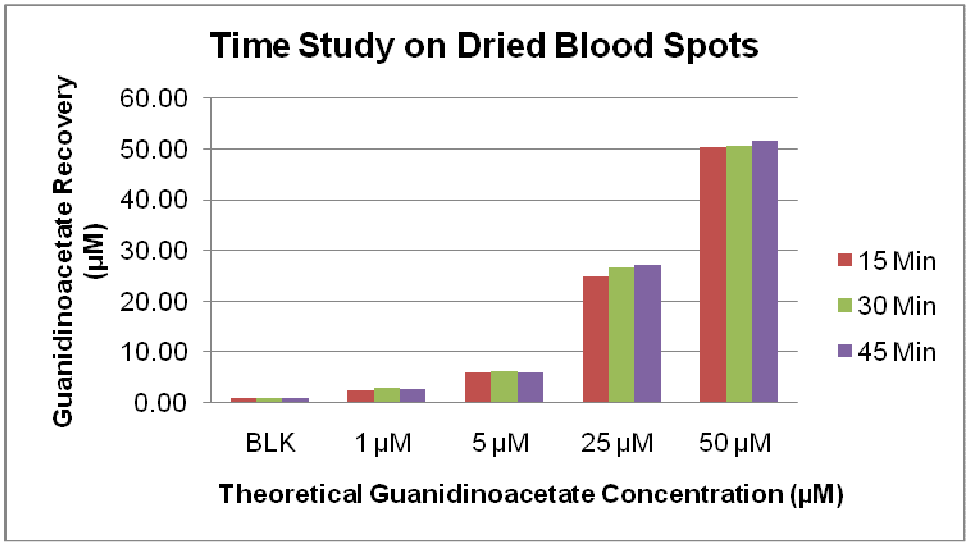


Figure 3. Guanidinoacetate standards prepared in blood.

This is the time routinely used for the extraction of acylcarnitines and amino acids. The solvent used for the extraction of guanidinoacetate is the same solvent routinely used for the newborn screening application.

Stability of Guanidinoacetate in Dried Blood Spots

For our study we used leftover blood spots, which had been stored for at least three months. We first assessed the stability of guanidinoacetate in dried blood spots stored at different temperatures (ambient, 2-8℃, and – 20℃ and lower). We used standards prepared in blood at several concentrations and spotted on filter paper. The dried blood spots, after drying for 24 hours at ambient temperature, were stored in a sealed bag with a desiccant, in the three temperature environments. The dried blood spots were tested in triplicate the day they were prepared, then daily for 3 days, weekly for 2 weeks, and monthly for 2 months. The results are shown in Figures 4-6.

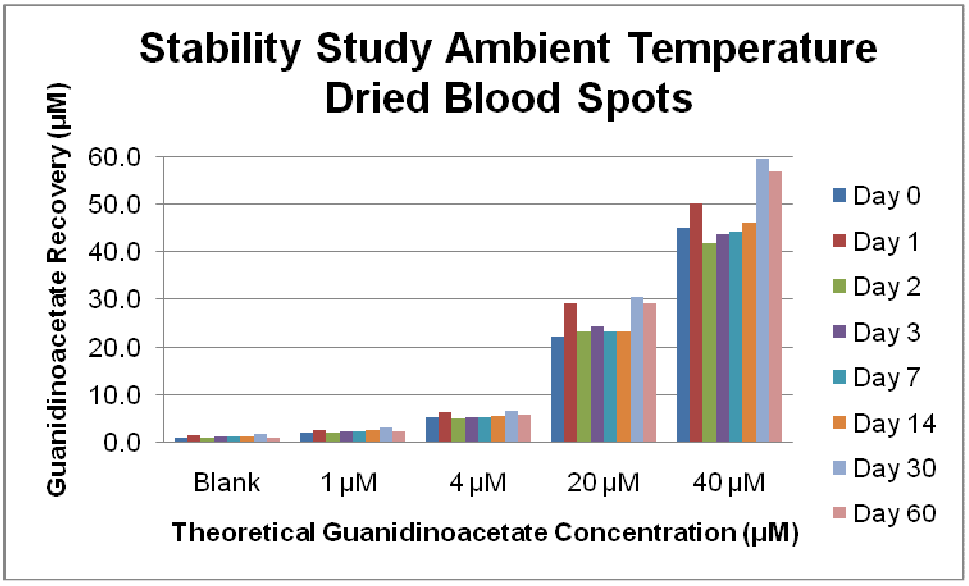


Figure 4. Dried blood spots spiked with guanidinoacetate and kept at room temperature.

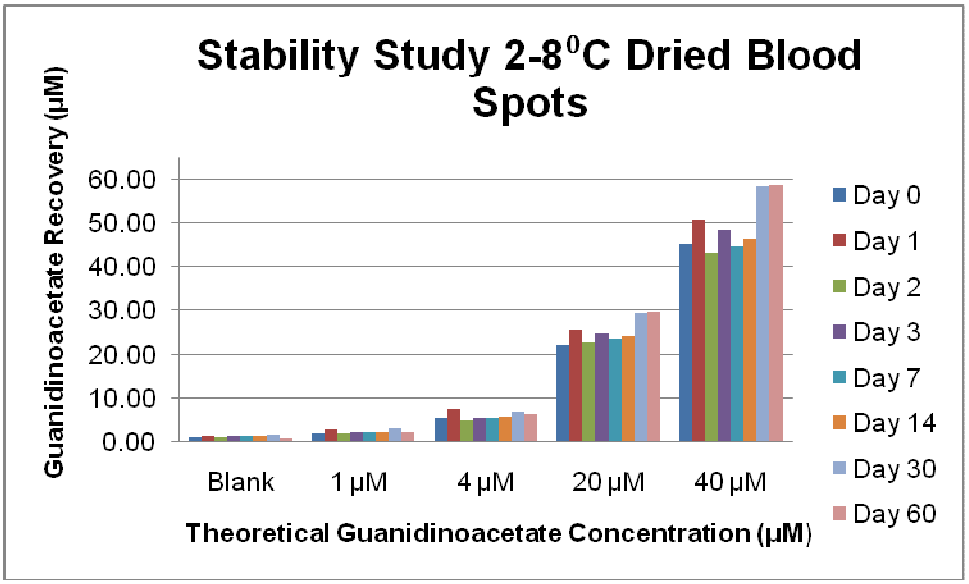


Figure 5. Dried blood spots spiked with guanidinoacetate and kept at 2-8ºC.

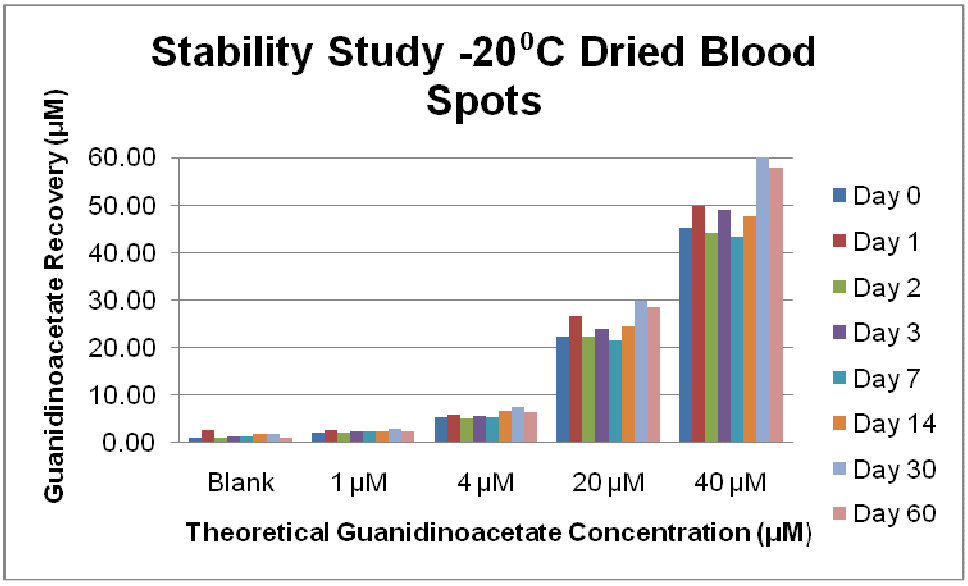


Figure 6. Guanidinoacetate recovery from dried blood spots kept at -20ºC.

The results obtained at different concentrations and temperatures do not show significant changes with time. Therefore guanidinoacetate is stable in the dried blood spots, for at least 2 months.

Analysis of Guanidinoacetate in Newborns' Blood Spots

To confirm that patients with GAMT deficiency and elevated guanidinoacetate could be identified by newborn screening using this method, we analyzed 163 previously-tested negative newborns' blood spots, deidentified by the Utah Department of Health according to their internal protocol, and one blood spot from a patient with GAMT deficiency (blood spots were obtained after parental informed consent, according to a protocol approved by the IRB of the University of Utah). A summary of the results obtained is shown in Table 1.

Table 1. Newborn Dried Blood Spot Summary

Healthy Population n=163			**Deficient Population n=1**		
Normal Newborns'	**Guanidinoacetate (μM)**	**Creatine (μM)**	**GAMT Patient #1**	**Guanidinoacetate (μM)**	**Creatine (μM)**
Average ±SD	1.42 (± 0.54)	506.95 (±142.4)	**1st Screen**	33.16	451.34
%CV	38.35	28.10	**2nd Screen**	10.45	167.06
Median	1.33	465.63			

SD is the standard deviation and %CV is the coefficient of variation.

The guanidinoacetate concentration determined in negative blood spots is significantly different (lower) than the concentration of the patient with GAMT deficiency, indicating that this method can be used to identify patients with GAMT deficiency at the time of their newborn screening. Table 1 includes GAMT patient results.

CHAPTER V

POLYMERASE CHAIN REACTION MATERIALS AND METHODS

Materials

Platinum Taq DNA Polymerase and 10 mM dNTP Mix were purchased from Invitrogen. ExoSAP-IT was purchased from Affymetrix. Lamda DNA/HindIII Marker 2 ladder was purchased from Fermentas. Dimethyl sulfoxide, ethylene glycol, and betaine (PCR grade) were purchased from Sigma Aldrich. The Failsafe™ PCR PreMix Selection Kit buffers were purchased from Epicentre Biotechnologies. All other reagents were prepared or used from an in house stock at ARUP Laboratories or the University of Utah.

GAMT

The *GAMT* Gene is composed of 6 exons on chromosome 19 (Nasrallah, et al., 2010) (Fig. 7) from base pair 1,348,087 to base pair 1,352,551. Chromosome 19 is gene rich with almost 1500 genes and has more than twice the gene density of the genome-wide average. Chromosome 19 was completely sequenced in 2004 and contains genes that code for diseases such as insulin-dependent diabetes, breast cancer, migraine headaches, and Alzheimer's (Gilbert, 2004).

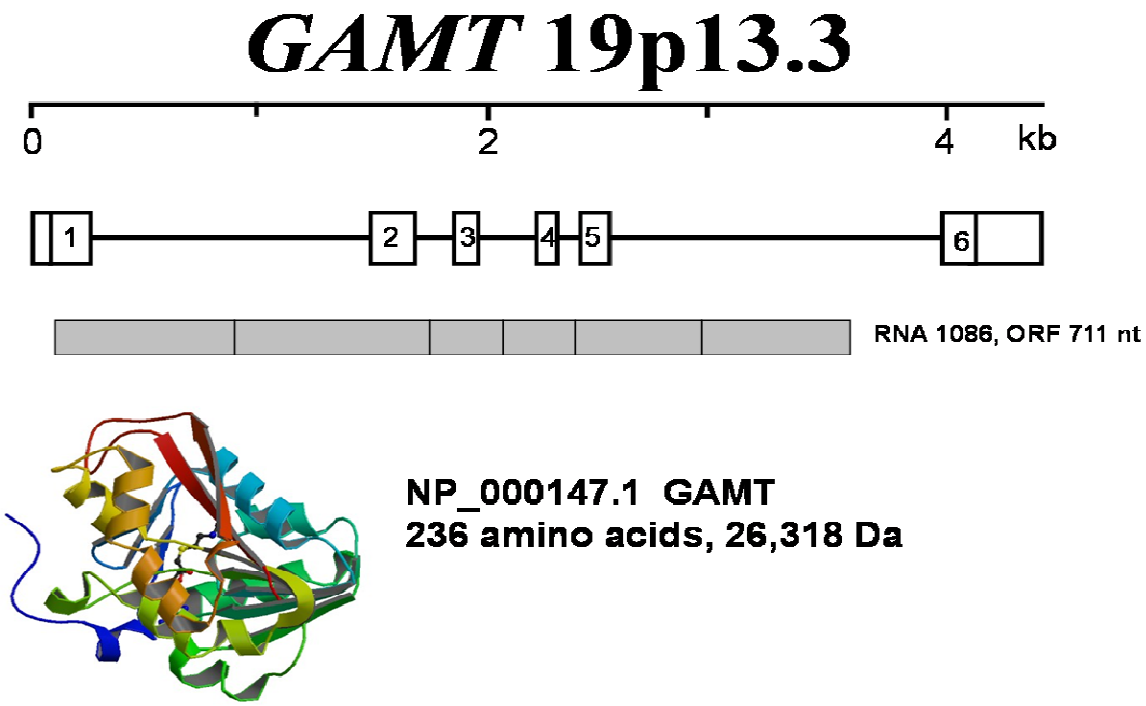


Figure 7. Guanidinoacetate methyltransferase: Schematic of the gene and the protein. (Longo et al., 2011)

GAMT provides the information for production of the enzyme guanidinoacetate methyltransferase. This enzyme is active in the liver, pancreas,and in the kidneys. The GAMT enzyme is a necessary component for the second step of the creatine biosynthetic pathway in which creatine is produced from guanidinoacetate (Verhoeven, et al., 2005).

Primers for the *GAMT* Gene's 6 exons were designed using the UCSC Genome browser and then checked with other online programs to avoid sites of single nucleotide polymorphisms and regions of homology with other genes. Exons 2 and 3 are very close and could be amplified in the same reaction. To these primers, M13 tails were attached to allow the sequencing of all PCR

products using the same M13 sequence as it is standard at ARUP Laboratories. Table 2 lists the primers with M13 tails.

PCR

The polymerase chain reaction (PCR) is an enzymatic process in which a specified region of deoxyribonucleic acid (DNA) is replicated numerous times to enable further studies (Sambrook, 2001). In the polymerase chain reaction, DNA is heated to separate the two strands of DNA, specific primers complementary to the DNA sequence are annealed at a lower temperature, and DNA is copied using the Taq polymerase capable of working at high temperatures (72ºC) and being resistant to the elevated temperatures needed to denature the DNA between cycles (>90ºC). This process is repeated for 30-40 cycles to generate copies of the desired sequence. The primers are the most important aspect for developing a PCR protocol. The two primers, forward and reverse, are short DNA sequences that flank the area that will be copied.

The polymerase chain reaction can be used to sequence the DNA of patients with genetic conditions. PCR can confirm or exclude their diagnosis (Item et al., 2004). For this project, PCR was used to amplify and sequence genes that can cause brain creatine deficiency.

For the *GAMT* Gene, DNA was used at 50 nanograms per microliter (final content was 2.5 nanograms per tube) in a standard reaction containing 10 mM dNTPs (final concentration 0.2mM each), 10x buffer (final concentration 1x), 50 mM magnesium chloride (final concentration 1.5mM), platinum Taq (final content

Table 2. *GAMT* Primers

Primer Pair	Amplicon Length with M13 Tail / base pairs	Forward Primer with M13 Tail (5'-3')	Reverse Primer with M13 Tail (5'-3')	GC %	Est. Tm
1	554	tgtaaaacgacggccagtcac tcccgccacctctc	caggaaacagctatgaccgtg aacgcctccgtgtg	71	58
2-3	579	tgtaaaacgacggccagtcag gcagcctcctaagcc	caggaaacagctatgacccca caagcaaaggagggg	67	60
4	211	tgtaaaacgacggccagtctg ggtgaggcgctgag	caggaaacagctatgaccaga ggggcttccccgag	71	61
5	586	tgtaaaacgacggccagtctct ctgacttgctgggatg	caggaaacagctatgaccagt acaggcacacgccac	55	57
6	329	tgtaaaacgacggccagtact cttcaatgaggggtggg	caggaaacagctatgaccgtg cgagaccctggactc	55	59

Primers were designed on July 2, 2010. M13 tails are in black. The GC column shows percent guanine-cytosine in the primers. The last column, Est. Tm, is for the estimated melting temperature.

1 unit per tube), nanopure™ water, and the forward and reverse primers at concentrations of 10 mM (final concentration of 0.2µM each). The protocol used was a denaturation step of 30 seconds at 94ºC, an annealing step for 30 seconds at 55ºC, and an extension time of 1 minute at 72ºC with a total of 30 cycles run using an Eppendorf Mastercycler Gradient (a PCR machine). Once the cycles were complete the PCR products were mixed with blue loading dye and put into a 1% agarose gel with ethidium bromide added. The gel was electrophoresed at 72 volts for 1 hour and the results were photographed with ultraviolet light.

The initial experiments generated nonspecific bands and occasional failed amplification. This required redesign of primer 6, the use of a different thermal cycler (Applied Biosystems GeneAmp PCR system 9700), the use of hot start,

touchdown, fail-safe buffers, and enhancing agents. These techniques and enhancers were used because the *GAMT* Gene and related primers are very guanine-cytosine rich, rendering the denaturation process more difficult (GC bonds require higher temperature for denaturation).

Hot start consisted of adding the polymerase after the DNA had been fully denatured. In a conventional polymerase chain reaction, the Taq DNA polymerase is active at room temperature and to a lesser degree, even on ice. In some instances, when all the reaction components are put together, nonspecific primer annealing can occur due to these low temperatures. This nonspecific annealed primer can then be extended by the Taq DNA polymerase, generating nonspecific products and lowering product yields. Adding the polymerase at time of the first annealing can increase yield and specificity of PCR (Sambrook, 2001).

Touchdown PCR is used to optimize PCR, increasing specificity, sensitivity and yield (Sambrook, 2001). With this method, the initial annealing temperature is higher than the projected melting temperature (T_m) of the primers being used, then progressively transitions to a lower, more permissive annealing temperature over the course of successive cycles. As the temperature approaches the one of the specific primer, this will be able to anneal to the correct sequence and initiate amplification, but not the amplification of other sequences, thus increasing the specificity. In the case of the *GAMT* Gene, all primer sets could be performed at the same time.

Failsafe™ buffers (Epicentre Biotechnologies) are pre-mixed buffers named A-L. Each of these buffers has a different mix of reagents. These buffers

function better at defined conditions including high guanine-cytosine content. With our *GAMT* primers, buffer G gave the best results.

A variety of PCR additives and enhancing agents have been used to increase the yield, specificity and consistency of PCR reactions. Whilst these additives may have beneficial effects on some amplifications it is impossible to predict which agents will be useful in a particular context and therefore they must be empirically tested for each combination of template and primers. Enhancing agents can stabilize the structure of DNA or the polymerase and increase amplification. Dimethyl sulfoxide (DMSO), betaine, ethylene glycol, and propanediol were investigated as enhancing agents. These agents have been used to increase product yield of primers that are guanine-cytosine rich (Zhang, et al., 2009). Problems arise with guanine-cytosine rich primers such as decreased separation of the strands from the numerous guanine-cytosine bonds, and possible guanine-cytosine intermolecular structure formations.

Results

Table 3 summarizes the PCR conditions and protocol that were developed to amplify all *GAMT* exons. The protocol was designed to allow all *GAMT* primers to work using the same conditions. Given the small size of the bands generated, a 2% agarose gel rather than the standard 1% was used to allow better movement of the small amplicons.

Figure 8 shows successful amplification of all amplicons (exons 2 and 3 were amplified in a single reaction) of the *GAMT* Gene. The expected amplicon

Table 3. PCR conditions and protocol for the amplification of the *GAMT* Gene.

GAMT PCR	**1X Mix**	**Final Concentration**		**Protocol**
FailSafe™ PCR 2X PreMix G	12.25 µL	1X		touchdown
Primer F 10mM	0.5 µL	0.2 µL	Hot Start	5 minutes at 95º
Primer R 10mM	0.5 µL	0.2 µL	Denaturation	30 seconds at 94º
template DNA 50 ng/µL	1 µL	2 ng	Annealing	45 seconds at 62º
Platinum Taq 5 U/µl	0.5 µL	1 unit	62-57º	Decrease in temp. each cycle until 57º
water	10.25 µL		Elongation	1 min at 72º
total	25 µL		total	32 cycles

length in base pairs is also shown. Samples were subsequently cleaned with Exosap, a single-step enzymatic cleanup of PCR products that eliminates unincorporated primers and dNTPs, and submitted to ARUP Laboratories sequencing facility for analysis. Figure 9 shows an example of the sequence obtained (Exon 1, with the ATG start site indicated).

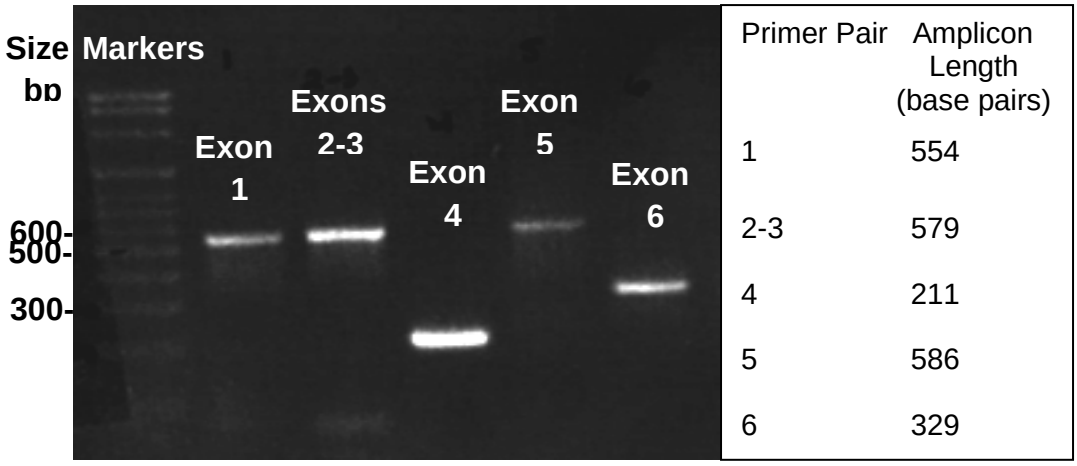


Figure 8. PCR amplification of exons 1-6 of the *GAMT* Gene. Primer pairs expected amplification location shown on right.

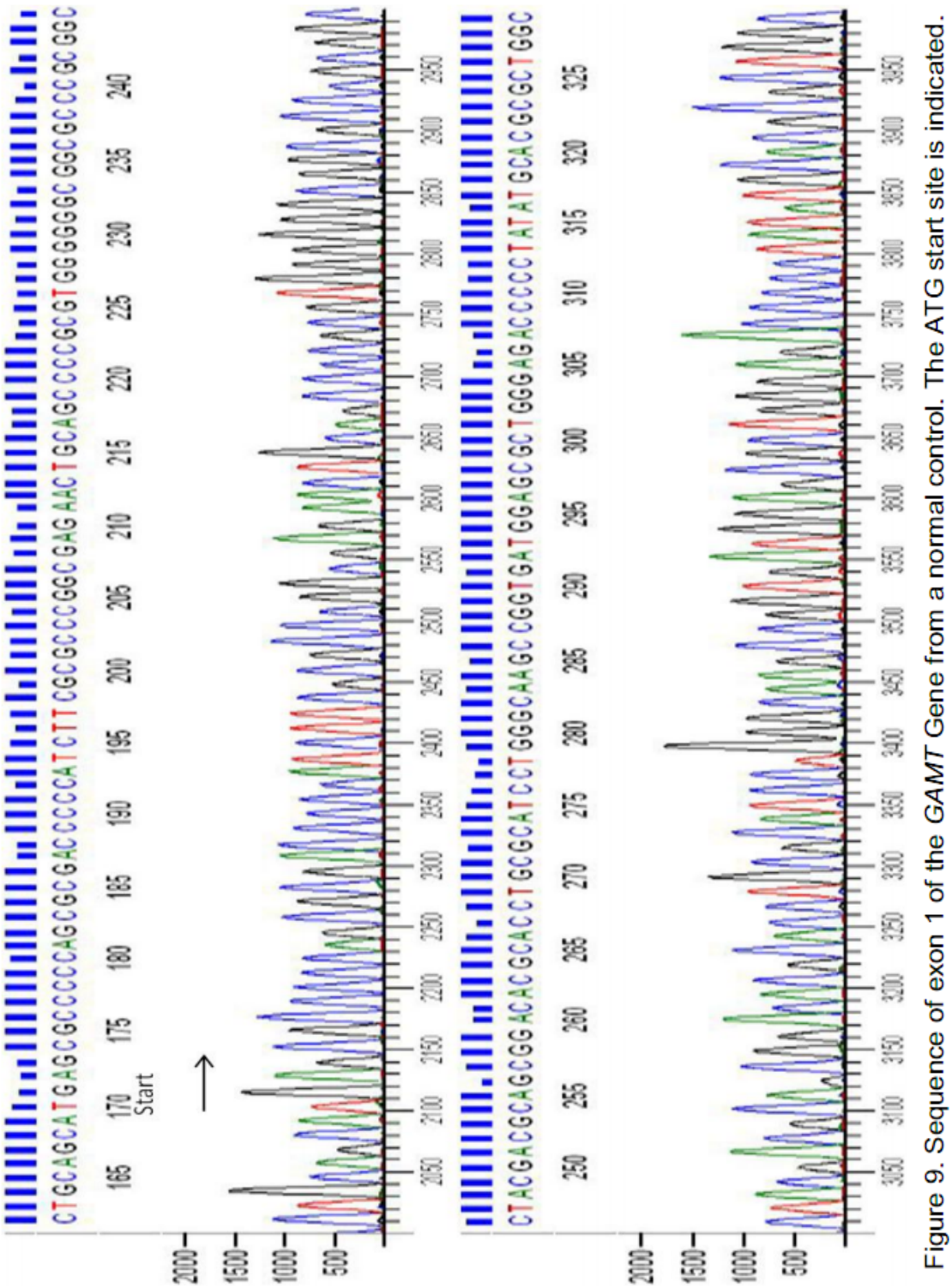


Figure 9. Sequence of exon 1 of the *GAMT* Gene from a normal control. The ATG start site is indicated.

GATM (*AGAT*)

Primers were developed for the *GATM* Gene (encoding the AGAT enzyme) in the same way as described for the *GAMT* Gene. The *AGAT* Gene is located on chromosome 15 (Edvardson, et al., 2010) and is 16,858 base pairs long (Item et al., 2001) (Fig. 10). It is located between base pairs 45,653,324 and 45,670,980. Exons 4 and 5 along with exons 6 and 7 were close and amplified together. Table 4 lists the AGAT primers with the M13 tails. Figure 11 shows PCR amplification of all exons of the *GATM* (AGAT) Gene with Failsafe buffer D that proved the most effective.

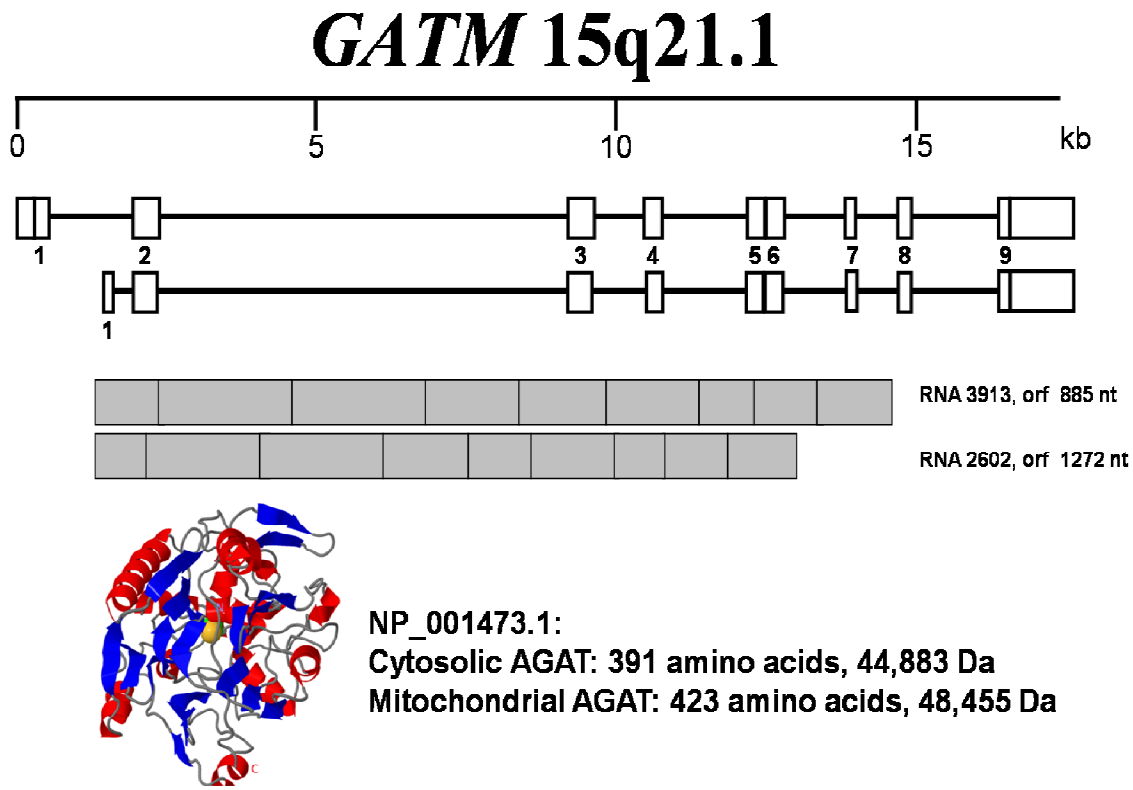
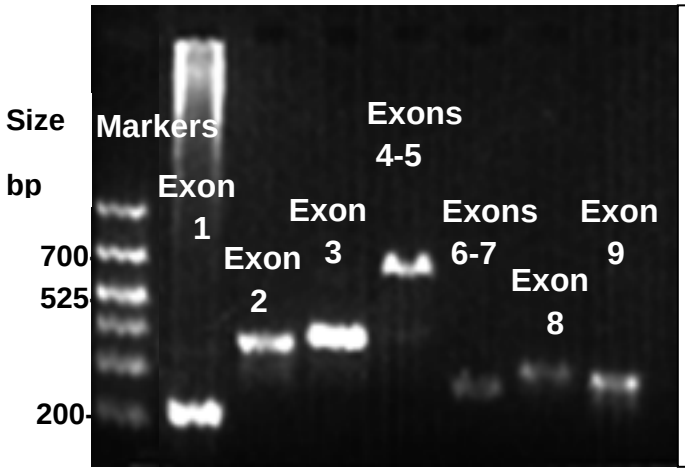


Figure 10. Arginine: glycine amidinotransferase: Schematic of the gene and the protein. (Longo et al., 2011) Crystal structure reconstructed with coordinates from (Humm, et al., 1997).

Table 4. *GATM* (AGAT) Primers

Primer Pair	Amplicon Length with M13 Tail/base pairs	Forward Primer with M13 Tail (5'-3')	Reverse Primer with M13 Tail (5'-3')	GC %	Est. Tm
1	226	tgtaaaacgacggccagtggaaggcttggaccgac	caggaaacagctatgacccgcaggatcgagtgagtc	65	57
2	394	tgtaaaacgacggccagtccatctcacttcctcctc	caggaaacagctatgaccagaggggtagcagcagcag	58	55
3	415	tgtaaaacgacggccagtgctgttactgcctatgaacc	caggaaacagctatgaccaaagcaaaggactctccaag	48	55
4-5	682	tgtaaaacgacggccagttttcttagtactgtatgccttatg	caggaaacagctatgacctcatttagaaccattsggaacc	32	54
6-7	292	tgtaaaacgacggccagtcagcttctcaaagagaattattactg	Caggaaacagctatgaccctaacatttgggctgctctc	35	56
8	336	tgtaaaacgacggccagtactgaaagaactgagctgtcac	caggaaacagctatgacctcaaacctagcatgtcatttc	45	55
9	313	tgtaaaacgacggccagtacaggactcctccaagtctg	caggaaacagctatgaccaagcaggagaatgaaccttg	55	55

Primers designed July 22, 2010 using UCSC Genome browser. M13 tails are in black.



Primer Pair	Amplicon Length (bp)
1	226
2	394
3	415
4-5	682
6-7	292
8	336
9	313

Figure 11. PCR amplification of all exons of the *GATM (*AGAT) Gene. Primer pairs expected amplification location shown on the right.

CHAPTER VI

DISCUSSION

GAMT and AGAT deficiencies are serious brain creatine deficiency disorders that result in mental retardation and seizures. The disorders may be under diagnosed due to nonspecific symptoms. These syndromes must be diagnosed as early as possible to prevent irreversible brain damage. Here we present a new three-tier testing method that is effective for testing dried blood spots for guanidinoacetate levels and could identify one of these syndromes (GAMT deficiency).

Significance of Findings

The primary screen by tandem mass spectroscopy was able to detect guanidinoacetate and creatine in dried blood spots at various concentrations. The second tier test confirmed quantitatively the abnormal guanidinoacetate in these samples. The third tier test, DNA testing, will further differentiate other causes of elevated guanidinoacetate from GAMT deficiency. This system can be easily included in the current screen, without the need to collect additional samples and with minimal additional cost. Only the cost of the internal standards for the additional analytes would be added to the existing cost of the screening in

addition to the cost of DNA testing. With this study, we have shown that there is no interference in the recovery of guanidinoacetate when extracting amino acids and acylcarnitines and, vice versa. The implementation of this screening would allow early treatment and improved outcome. The *GAMT* and *GATM* Gene primers developed here could also be used for sequencing these two genes for clinical purposes.

Limitations and Future Work

The next step is to optimize DNA extraction from leftover newborn dried blood spots. Each punch from a dried blood spot, depending on the size, contains 3-8 µL of blood. Methods are available for efficient extraction of DNA from these small samples, and only a small amount of DNA is required for the polymerase chain reaction; however, the process must be validated.

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